Physico-chemical Analyses of the Humoral Immune Response to HIV-1: Quantification of Antibodies, Their Binding to Viral Antigens and Neutralization of Viral Infectivity

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Antibodies against HIV-1 envelope glycoproteins (Env) as well as the Gag and other viral proteins are produced in the course of the HIV-1 infection. The isotypic composition differs between the antibody response to the Gag and the Env proteins. Falling plasma levels of antibodies directed to the Gag proteins are associated with progression of HIV-1-induced disease. Yet, it is antibodies directed to the Env protein that neutralize viral infectivity. How can we quantify these kinds of antibodies and predict their degree of complex formation with antigens *in vivo* and *in vitro*? How should we define, measure or calculate affinity, association and dissociation constants, on- and off-rate constants, and binding energy? What does the titer of a serum or plasma reveal about antibody affinity and concentration? How are antigenic peptides related to epitopes? What can antibody binding tell us about the tertiary and quaternary structure of HIV-1 proteins? How is viral neutralization best measured in relation to the binding of neutralizing antibodies? What can be the structural and functional basis of HIV-1 resistance to neutralization? These problems are discussed against the background of different derivations from the law of mass action.

Introduction

Antibodies are both the tools and object of study in much HIV-1 research. The analysis of their binding treads on a narrow path between quantitative errors and interpretative flaws. An understanding of how antibodies bind to virus particles is central to inquiries into their neutralization of viral infectivity and the viral escape from such effects. The latter aspects of HIV-1 have recently been comprehensively reviewed (Moore and Ho 1995; Poignard et al. 1996; Sattentau 1996), and will only be referred to here for illustrative examples of reasoning about antibody binding and neutralizing mechanisms.

Some efforts to unravel the pathogenesis of AIDS focus on the prevalence of anti-HIV-1 antibodies and viral antigens in the plasma of infected persons. The turnover of replicating HIV-1 is considerable even in the clinically quiescent phase of the infection (Ho et al. 1995; Wei et al. 1995). Eventually, viremia and antigenemia rise and antibody levels fall. In particular, declining titers of Gag-specific antibodies herald clinical deterioration (Lange et al. 1986; Goudsmit et al. 1987; Weber et al. 1987; Binley et al. 1996). Therefore, determination of the concentrations of antibodies and antigens, and of their degree of complex formation, may assist in dissecting cause from effect: is a decline in antibody concentration in the blood physico-chemically explicable as a result of rising antigenemia?

The HIV-1 envelope glycoprotein consists of gp120, the surface moiety, or SU, which is bound to gp41, the transmembrane protein, or TM (Weiss 1993). There is evidence that these hetero-dimers form homo-trimers (Blacklow et al. 1995; Fass et al. 1996), but their structure is not known. However, three-dimensional structural information is available for some antibody binding sites on both SU (Ghiara et al. 1994) and TM (Oldstone et al. 1991; Klasse et al. 1993). Furthermore, antibodies have been used to probe the structure of the SU protein (Moore and Sodroski 1996). Here, the methodological basis for inferences of protein-structural relationships from antibody-binding evidence will be discussed.

Isotypes of the antibody response to HIV-1

Antibodies not only bind specifically to antigens but also recruit other molecules and cells in the immune attacks on pathogens. These separate functions are carried out by different parts of the antibody molecule. Most of the following discussion will be concerned with the antigen-binding portions, which consist of the variable domains of the heavy and light chains. The antigen-binding function is retained by each of the two Fab fragments that result from proteolytic cleavage of a bivalent antibody (Janeway 1996). It is the other end of the molecule, the Fc portion, that binds to complement factors and cellular receptors. The Fc portion consists entirely of domains of the heavy chains.

The heavy chain determines which isotype, *i.e.*, class or subclass, to which an immunoglobulin molecule belongs. The isotypes interact differently with humoral and cell-associated accessory molecules. In humans there are five immunoglobulin classes, IgM, IgG, IgA, IgD and IgE. IgG has four subclasses: IgG1, IgG2, IgG3 and IgG4. IgA has two: IgA1 and IgA2. IgG3 and IgM are the most potent activators of complement via the classical pathway. IgG2 and IgG4 pass the placental barrier most efficiently, while IgG1 and IgG3 preferentially bind to monocytes and macrophages via the Fc receptors on these cells (Janeway 1996).

It is thus conceivable that the isotypes of antibodies not only influence their half-life in plasma and local concentrations in various tissues and organs, but also affect their potential to enhance or impede virus infection *in vivo* and *in vitro*. Because of the different numbers of antigen-binding sites on antibodies of different isotypes, *e.g.* ten on pentameric IgM, four on dimeric IgA and two on IgG, binding properties are influenced by isotype as well.

Furthermore, isotypic analyses of the antibody response to the virus may assist in the staging of clinical HIV-1 infection: in the ontogenesis of a particular antibody, development of increased antigen binding capacity occurs in parallel with isotypic switches. For example, B-cells originally secreting IgM with weak binding of each Fab to a certain antigen may evolve to produce IgG or IgA with stronger binding of each Fab group (Janeway 1996). This development can be mimicked *in vitro*. Peripheral blood mononuclear cells from an HIV-1-negative individual were stimulated *in vitro* to secrete IgM specific for a site on the SU of the HIV-1 Env protein. Subsequently, the same cells were induced to secrete IgG by co-culture with immunized T-cells and the provision of the necessary signals *in vitro* (Chin et al. 1995). *In vivo*, this process is potentially modulated by the HIV-1 infection: it is partly regulated by T helper cells, a major target of the virus.

What is the clinical significance of the isotypic pattern of HIV-1-specific antibodies? A more narrow isotypic profile of anti-HIV-1 antibodies was observed in sera from AIDS patients than from asymptomatic carriers of HIV-1: this was seen both by analyses with whole-virus ELISA (enzyme-linked immunosorbent assay) (Sundqvist et al. 1986), and when the antibody response was dissected with regard to antigen-specificity and isotype by Western blot (Klasse and Blomberg 1987; Khalife et al. 1988; Broliden et al. 1989), probably as a reflection of a general decline in the antibody levels with terminal clinical progression (McDougal et al. 1987).

The dominant isotype in the anti-HIV-1 response is IgG1, in particular of the Env-specific antibodies (Klasse and Blomberg 1987; McDougal et al. 1987; Khalife et al. 1988; Mathiesen et al. 1989). IgG1 is also the major subclass mediating antibody-dependent cellular cytotoxicity of HIV-1-infected cells (Ljunggren et al. 1988). IgG2 directed to Env was detected with a synthetic peptide that contains a potential N-linked glycosylation site (Chiodi et al. 1989). Some IgG2 responses to carbohydrate antigens are T cell- independent, but the extensive carbohydrate moieties of the Env protein do not yield a substantial IgG2 response (Klasse and Blomberg 1987; McDougal et al. 1987; Khalife et al. 1988).

IgG3 is directed mainly to the Gag protein of the virus and in particular to the matrix protein p17 (Klasse and Blomberg 1987; McDougal et al. 1987; Khalife et al. 1988). The IgG3 reactivity is more frequent in early than in late clinical stages (Klasse and Blomberg 1987; McDougal et al. 1987; Ljunggren et al. 1988). The near restriction to IgG1 of the anti-Env response, and the prevalence of IgG3 directed against Gag, may reflect different regulatory mechanisms of the antibody responses to these proteins. Differential regulation might explain why total anti-Gag antibody concentrations tend to

decline earlier than those of Env-specific antibodies (Lange et al. 1986; Goudsmit et al. 1987; McDougal et al. 1987; Weber et al. 1987; Binley et al. 1996).

IgG4, which has been detected mainly against Gag proteins, is more common in hemophiliac than in other HIV-1-infected subjects (Klasse and Blomberg 1987; Khalife et al. 1988; Klasse et al. 1988). This could be because of longer duration or different modes of infection in the hemophiliacs than the other subjects: IgG4 tends to occur after prolonged or iterated exposure to an immunogen (discussed in Khalife et al. 1988; Klasse et al. 1988), and has indeed been observed somewhat more often in sera obtained late in the clinical course of HIV-1 infection (Ljunggren et al. 1988; Mathiesen et al. 1989). Likewise IgE, directed to Gag, was preferentially detected in hemophiliac subjects (Khalife et al. 1988). In quantitative studies of antibodies, isotypes matter because valency affects binding characteristics. In binding assays for human sera, isotype-specific or cross-reactive antibodies for detection are chosen in accordance with the object of study, which is often the four subclasses of IgG.

The structures of antibody-HIV-1 recognition

Antigenicity and Immunogenicity. Antigenicity is the property of being recognized by an immune response; immunogenicity is the capacity to elicit one. In the simplest case, the molecule used for the immunization, the immunogen, is better recognized, when used as an antigen in a binding assay, than any related molecule. But this may not be so, *e.g.* if a protein immunogen is denatured or cleaved in the immunized organism. Forms of the molecule thus modified may then be more antigenic than the original immunogen. Haptens are molecules that must first be conjugated to carrier molecules in order to elicit the production of antibodies, which may recognize the unconjugated molecule: haptens are antigenic without being immunogenic (Janeway 1996).

Paratopes. The amino-acid sequences of the variable domains of both heavy and light chains comprise three hypervariable regions intercalated between more conserved stretches, which are termed framework regions. The hypervariable regions are crucial in creating the specific antigen-recognition surfaces: hence they are also known as the complementarity determining regions, or CDRs. The paratope is the surface of the Fab that makes contact with the antigen, through non-covalent bonding (Janeway 1996). The derived amino-acid sequences, and mutations in them, from some HIV-1-specific variable domains have been described (Binley et al. 1996; Thompson et al. 1996; Watkins et al. 1996).

Epitopes. Strictly defined, epitopes are the surfaces on antigens that make contact, *i.e.*, are engaged in non-covalent bonding, with the paratope. An epitope is thus a three-dimensional patch of surface-accessible atoms on a folded protein. When this patch contains residues that are all contiguous in the amino-acid sequence of the protein, the epitope is called continuous. Epitopes formed by residues that are far apart in the primary sequence are named discontinuous. What surfaces on proteins are made up exclusively of residues that are contiguous in the primary sequence, and thus could possibly be represented by a synthetic peptide? Hardly any surfaces of the size of empirically measured epitopes are composed entirely of contiguous residues (Barlow et al. 1986). A peptide may represent enough of a nearly continuous epitope, or a large segment of a discontinuous one, so that the peptide is recognized by the antibodies that are directed to the epitope on the native protein. Those exposed parts that are most likely to have the majority of their residues in primary-structural contiguity consist of loops and turns. Indeed, it is peptides corresponding to loops and turns in the protein structure that are most likely to cross-react with the native protein; the successful use of hydrophilicity plots as a basis for choosing cross-reactive peptides can largely be attributed to the frequent co-incidence of a high degree of primary-structural continuity, surface exposure, flexibility and hydrophilicity (Lerner 1982; Tainer et al. 1984; Barlow et al. 1986; Colman et al. 1987; Colman 1988). As an exception to this rule, a hydrophobic peptide derived from the external portion of HIV-1 TM frequently reacts with HIV-1-positive sera (Narvanen et al. 1988).

Whether the entire epitope or a part of it is simulated by the peptide, there is still the problem of shape. Many short peptides are disordered in solution, while the corresponding part of the protein is under the constraints of the tertiary structure of the folded protein (Creighton 1993). How then can the peptide fit the same paratope as the protein epitope? Or if a peptide was used as the immunogen, how can the antibodies elicited recognize the corresponding amino-acid stretch in the native protein (Lerner

1982; Tainer et al. 1984; Doolittle 1986)? These cross-reactions, in both directions, may be due to a flexibility that allows a remolding of the peptide or stretch of polypeptide chain, to create a better fit to the shape of the paratope (Doolittle 1986; Creighton 1993).

Three-dimensional structures of Env epitopes and quaternary-structural influences on anti-genicity. Antibody-reactive peptides obviously contain antigenic structures (Klasse et al. 1991): whether we call these epitopes is immaterial as long as it is remembered that the epitope on the native protein is unlikely to be identical (Laver et al. 1990). In fact, small deviations in sequence in a peptide antigen from the sequence of the immunogen can sometimes fortuitously increase antigenicity, as illustrated by systematic variation of TM peptides (Klasse et al. 1991). The structure of some antigenic peptides derived from the extravirional part of TM has been determined by nuclear magnetic resonance: some such peptides that are α-helical in solution (Wild et al. 1992; Klasse et al. 1993) correspond to the carboxy-terminal segment of longer peptides that form a coiled coil (Wild et al. 1994). A peptide which is immediately carboxy-terminal in sequence to the α-helix, contains a disulfide bond and a type I reverse turn (Oldstone et al. 1991). However, these regions may have different structures in the native protein before or after complexing with antibodies.

The amino-terminal region of TM that has a propensity to form α helices and coiled coils (Wild et al. 1992; Klasse et al. 1993) is necessary for oligomerization of TM and thereby possibly of the whole SU-TM complex (Wild et al. 1994; Poumbourios et al. 1995). Deletions in the amino-terminal half of the extravirional portion of TM that interfere with oligomerization of Env also decrease the antigenicity of an epitope in a more carboxy-terminally located region (residues 634 to 664) (Poumbourios et al. 1992). Affinity-purified human antibodies to a region overlapping with the putative coiled coil were shown to recognize oligomeric TM preferentially, while, in contrast, a murine MAb, which recognizes a sequence within the same region, reacted more strongly with the monomeric protein. Immunization with a soluble oligomeric form of Env elicited mainly oligomer-specific antibodies directed to TM, while SU induced a more monomer-specific response (Broder et al. 1994).

The hetero-oligomerization between SU and TM generally reduces the antigenicity of most TM epitopes: when SU is made to dissociate from TM by incubation with soluble CD4, these epitopes become better exposed. But one human TM-reactive MAb, 2F5, binds more weakly after dissociation of SU (Sattentau et al. 1995). This MAb recognizes a short peptide (ELDKWA, corresponding to residues 667–672) (Purtscher et al. 1994). Human sera differ in their recognition of peptides with overlapping sequences derived from the region around the recognition site of 2F5: an antigenic structure with more frequent sero-reactivity was identified on the carboxy-terminal side of ELDKWA (Calarota et al. 1996). However, when human Fab fragments directed to HIV-1 TM were isolated by panning of phage-displayed antibody libraries (Binley et al. 1996), the majority of the Fabs recognized the region 649–668 (*cf* Robson et al. 1987), located immediately amino-terminally to the ELDKWA motif. Fewer Fabs recognized a structure in the region of residues 584–609. That the TM epitopes in both of these groups were conformationally dependent could be due to the selection procedure that involves panning against native antigen (Binley et al. 1996). This may also explain why previously a greater proportion of human MAbs, isolated by classic cloning, recognized a structure on reduced TM in the region around the two cysteine residues 598 and 604 (Xu et al. 1991).

The structure of a peptide derived from the V3 region of SU in complex with a paratope has been solved by crystallography: 10 central residues in a 26-residue peptide were inflexible enough in the complex to allow interpretation of the electron-density map. The tip of the V3 loop, GPGRAFY, assumed an S-shape including a type II, a type III and a type I β turn (Ghiara et al. 1994). It is an open question whether this well-defined secondary structure is present in native SU or is induced by the antibody that binds both to the peptide and to oligomeric SU on virus particles. Whether atoms from residues outside the peptide sequence contribute to the native-protein epitope (cf. (Seligman et al. 1996)) may only be answered by the crystallographic analysis of the Fab bound to the whole protein. Other examples from studies on the V3 region of HIV-1 SU reinforce the need to think of all epitopes as three-dimensional surfaces complementing the corresponding composite structures on the paratopes. By the use of phage display of antibodies, the light chain of a MAb that binds to the V3 region of SU of the IIIB strain was exchanged. The new MAb retained the ability to bind to V3 of IIIB, but unlike the

original MAb also recognized strains with different residues on the amino-terminal side of V3. Neither antibody bound to SU that differed from IIIB on the carboxy-terminal side of V3 (Watkins et al. 1993).

One human MAb recognized V3 peptides with the MN, SF-2, HxB2 and BH10 sequences equally well, bound strongly to SF-2- and MN- and weakly to HxB2-SU monomers, but it failed to recognize monomeric BH10 SU at all. Another human MAb bound to SU of the isolate AD-6, and to the corresponding peptide in solution but not in solid phase. Furthermore, serum originating from the same individual as the AD-6 isolate reacted more strongly with the MN V3 peptide than with the AD-6 V3 peptide in a solid-phase assay (Moore et al. 1994). Generally, the antigenicities of peptides with the V3 sequences of SF-2, MN, and IIIB were shown to differ drastically between solution and solid-phase conditions (Moore et al. 1993).

The V3 epitopes on monomeric and oligomeric Env differ both in antigenic and immunogenic properties. An oligomeric form of truncated, recombinant Env elicited antibodies in mice which preferentially recognized the non-reduced form of TM, although the response to SU was less conformation-sensitive. However, only a small fraction of the MAbs derived from these mice recognized V3, while a large proportion of MAbs raised against monomeric Env was directed to V3 (Earl et al. 1994). Binding of a range of antibodies to SU from T-cell line-adapted HIV-1, with the exception of those directed to V3, was stronger to monomeric than to oligomeric Env (Sattentau and Moore 1995). The oligomeric form of primary-isolate Env may shield even the V3 region from antibody binding (Bouhabib et al. 1994).

These examples of differences between oligomers, monomers and peptides as antigens show the need to distinguish between weak and strong binding. The concept of affinity is required to understand these differences.

Quantification of antigen-antibody binding

The association of a Fab with an an antigen follows the law of mass action (Creighton 1993). The reaction of Fab A and the antigen B can be written

$$A + B \rightleftharpoons AB.$$

The two arrows mean that the reaction is reversible: some of the complexes formed will dissociate, but to begin with more and more complex will accumulate. The law states that as an equilibrium is approached, the ratio of the concentration of complex, $[AB]$, over the product of concentrations of free reactants, $[A][B]$, will approximate a constant. This is the association constant, K_a, which is characteristic for the propensity of these two molecules to bind

$$K_a = \frac{[AB]}{[A][B]}.$$

The propensity to dissociate can be quantitatively described by the dissociation constant, K_d:

$$K_d = \frac{[A][B]}{[AB]}.$$

It is readily seen that K_d is just the reciprocal of K_a

$$K_d = \frac{1}{K_a}.$$

The higher the *affinity* of A for B, the greater the association constant, K_a. However, as we have seen, the affinity is equally well described by the dissociation constant, K_d: the lower the K_d, the higher the affinity. The two constants provide the same information but must not be confused, and the redundant term "affinity constant" should be used synonymously with *association constant*, if at all.

Avidity. So far we have considered monovalent Fabs as the binding agent. How should we reason about bivalent antibodies, for example IgG? Although the paratope in the context of the whole IgG molecule has the same affinity for a monovalent antigen in solution as the Fab, the binding to antigen molecules with more than one epitope, or to monovalent antigen immobilized to solid phase, is potentially much stronger. It cannot rationally be predicted how much stronger the bivalent than the monovalent binding of the IgG is, because this would depend on the orientation and distance of adjacent epitopes to each other, as well as on the flexibility of the IgG. The bivalent antibody is said to have a higher *avidity* (Janeway 1996) for the polyvalent antigen than the Fab does. However, while the affinity can be quantitatively expressed, the avidity is not well defined. It is theoretically attractive is to consider the *functional* affinity (Underwood 1988) of the paratope in the context of the Fab or the entire IgG, when binding to either monovalent or polyvalent antigen. The avidity could thus be defined as the ratio of the K_d for the monovalent reaction over that for the polyvalent reaction. The problem is that in an ELISA, one does not distinguish between the three different binding states of a bivalent IgG: one paratope bound, the other paratope bound, and both paratopes bound. However, the two-point binding of the antibody is the most probable state when the spacing of the epitopes is favorable. Thus, we might define avidity as the ratio of monovalent over polyvalent K_d, regardless of state of binding.

The dissociation constant, K_d, has the dimension of concentration: it can be expressed in units $[M]$. Consider the situation where A and B are present at such concentrations that, at equilibrium, half of the antigen molecules will be complexed with paratopes: this means that the concentration of free $[B]$ is equal to the concentration of complexes, because half of the original concentration of B has been used up to create AB. Then we can substitute $[AB]$ for $[B]$ in the equation for K_d

$$K_d = \frac{[A][AB]}{[AB]},$$

thus $K_d = [A]$.

In other words, the concentration of free $[A]$ is now equal to the dissociation constant, K_d. This provides a method of measuring the affinity. Mathematically it corresponds to the negative reciprocal of the constant that determines the slope of a curve in a Scatchard plot, which is the ratio of the concentration of complex over free $[A]$ as a function of the concentration of complex $[A]$ (Creighton 1993). However, few Scatchard-plot measurements are available for HIV-1 antibodies or ligands.

A short-cut to a sufficiently good approximation is suggested by the equations: when the *initial* concentration of antigen is much lower than the *initial* concentration of paratopes, it follows that the maximal amount of complex formed must be much lower than the amount of free paratopes at equilibrium, $[AB] \ll [A]$; thus the total concentration of A will be a good approximation of the concentration of free A. Therefore, the concentration of total A that is required to bind to half of the antigen molecules approximately equals the dissociation constant, K_d. Hence the measurements of concentrations of ligands that give half-maximal binding can give a good estimate of the affinity, provided that two conditions are fulfilled:

1) The amount of ligand that is titrated is in great excess over the molecule it binds, *even in the zone of half-maximal binding*.
2) Half-maximal signal, (*i.e.* optical density (OD)), in the assay corresponds to an occupancy of half of the antigen molecules; and the linear relationship between the amount of complex formed and the specific signal in the assay has been demonstrated independently.

Relative occupancy, here called θ, of the antigen that results from the binding of paratopes $[A]$ at a certain concentration, can be derived from the law of mass action (for a derivation, see Klasse and Moore 1996):

$$\theta = \frac{([A]/K_d)}{(1 + [A]/K_d)}.$$

Sometimes it is known that the amount of antigen bound to the solid phase in an ELISA is negligible compared with the amount of antibody present at half-maximal binding. Then the binding curve should

approach the ideal sigmoid shape illustrated in Figure 1. However, we can check whether different values on the curve conform to the law of mass action, granted the assumptions of antibody excess. If they do, that lends credence to the half-maximal binding concentration as a good approximation of K_d. If they do not, we can find out what relation the concentration that yields half-maximal binding bears to the real K_d. It should not be expected that the approximation of total to free antibody can be upheld in the lowest range of concentrations, far below K_d. Therefore it is advantageous to perform the following tests on OD values above the half-maximal value.

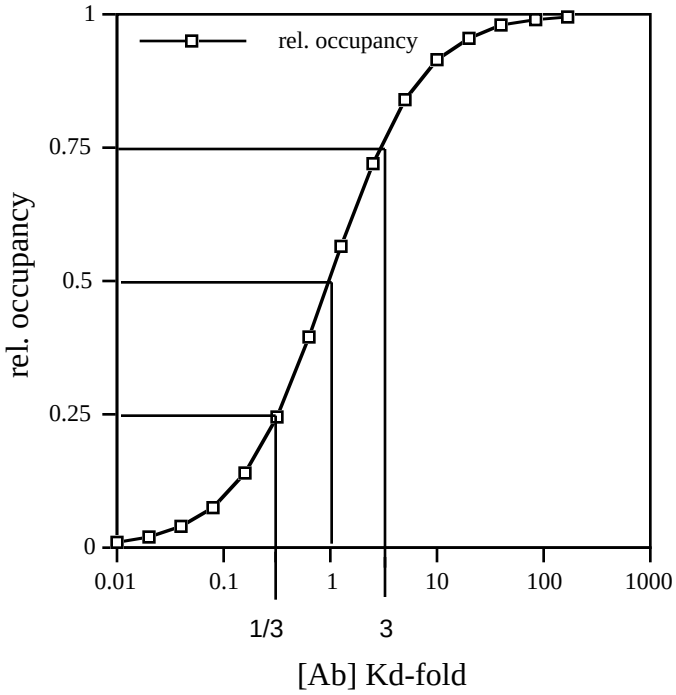

Figure 1. An idealized example is given of the mathematical analysis of antibody binding in an ELISA. Here, both the antibody concentration and the dissociation constant, K_d, for the antibody binding to solid-phase antigen are known. The concentration of free antibody, $[Ab]$, relative to the K_d, in K_d-fold, is plotted on the x axis, and a measurement of the relative occupancy, q, such as the OD of the ELISA, on the y axis. The law of mass action predicts a sigmoid curve describing the relative occupancy on the antigen as a function of $[Ab]/K_d$ in accordance with the formula $\theta = ([Ab]/K_d)/(1+[Ab]/K_d)$. A relative occupancy of 0.5 is obtained when $[Ab]/K_d = 1$, *i.e.*, when $[Ab] = K_d$. With the antibody in large excess of the antigen, the concentration of free antibody is approximately equal to the total concentration of antibody. If this approximation is not justifiable, the ratio of the $[Ab]/K_d$ value at 0.5 and 0.75 will be less than 3. By checking this and other theoretically predicted values, the antibody excess and the agreement with the law of mass action can be tested.

Let:

K_d = the dissociation constant for IgG molecules binding to solid-phase-anchored antigen: an IgG molecule is regarded as dissociated when neither of its paratopes remains bound.

$[Ab]_{0.5}$ =the total concentration of antibodies specific for the HIV-1 antigen in question at the relative OD of 0.5

$[Ab]_{0.75}$ = the total concentration of antibodies specific for the relevant HIV-1 antigen at the relative OD of 0.75.

$[AbBS]$ = total amount per volume of solid-phase-anchored antibody-binding sites (epitope pairs). In order to simplify, this is regarded as a concentration, or formally as a thermodynamic activity like the other concentrations (Atkins 1986).

Then, according to the law of mass action, at equilibrium we have

$$\theta = \frac{([Ab]/K_d)}{(1 + [Ab]/K_d)},$$

where θ now is the relative occupancy by bivalent antibodies on the solid phase-anchored antibody-binding sites $K_d = [Ab]_{0.5} - 0.5[AbBS]$ and $3K_d = [Ab]_{0.75} - 0.75[AbBS]$. By elimination of $[AbBS]$ from these two equations and introduction of the factor $q = [Ab]_{0.75}/[Ab]_{0.5}$ we get

$$K_d = \big((2q/3) - 1\big)[Ab]_{0.5}.$$

We can now check that at the theoretically ideal value $q = 3$, we do indeed get $K_d = [Ab]_{0.5}$. Obviously, corrections can also use values for other theoretically predicted relationships of K_d to concentrations giving defined occupancies, for example $9K_d = [Ab]_{0.9} - 0.9[AbBS]$. The source of aberration we considered can only lead to overestimates of K_d. It is also clear that when great aberrations occur, such as with $q < 2$, the curves should not be used for K_d estimations. An absolute requirement is that there is some excess of antibody over binding sites even around half-maximal binding. Other kinds of aberrations of the curve from the ideal may be due to stark affinity heterogeneities among antibody populations in the serum. Such aberrations cannot be corrected by this method and would preclude further analysis.

In summary, simple titrations of antibodies or sera can provide an excellent method for determining an approximate value of K_d or a dilution at which the relevant antibodies would be present at concentrations close to their average K_d.

The meaning of antibody titers: a relationship between affinity and concentration. A common feature of clinical HIV-1 research is the determination of serum or plasma titers against HIV-1 antigens in ELISAs. We have seen that under certain conditions the concentration of a pure MAb that gives half-maximal binding is an estimate of K_d. But when the antibody concentration is not known, and when the serum contains a mixture of antibodies, what does the titer, the dilution that gives half-maximal binding, mean? The answer is incontrovertible: titers depend on a relationship of antibody concentrations to their affinities, as shown by the expression for occupancy

$$\theta = \frac{([Ab]/K_d)}{(1 + [Ab]/K_d)}.$$

One approach to determining the concentration in a human serum of antibodies directed to a certain epitope on SU has been to measure the competition between the serum and a labeled MAb for binding to SU. However, the binding capacity of the polyclonal serum can then only be expressed as MAb equivalents, *i.e.*, what the concentration would be if the antibodies had the same affinity as the MAb (Moore et al. 1994). The following approach is an attempt to extract specific information about concentration and affinity from the composite phenomenon of a serum titer.

Suppose we have determined the dilution at which the antibodies directed to a solid-phase HIV-1 antigen are present at concentrations close to their K_d, as above. We now incubate a defined dilution of this serum with a range of concentrations of the same antigen in soluble form. This takes place in wells that also contain the antigen in its solid-phase anchored form (Binley et al. 1996). A double equilibrium between antibody binding to soluble and solid-phase antigen will be approached.

Let:

A = total paratope concentration

A_{free} = concentration of unoccupied paratopes

B = total soluble antigen concentration

$C =$ concentration of complex between paratope and epitope in solution

$K_{d(s)} =$ the dissociation constant for the binding of paratopes to epitopes on antigen in solution

$E =$ solid phase-anchored epitope-paratope complex

$F =$ total solid phase-anchored epitopes

$K_{d(sp)} =$ the dissociation constant for the binding of paratopes to epitopes on antigen that is anchored to solid phase.

We apply the law of mass action to double equilibria (Creighton 1993; Klasse and Moore 1996). This means that the concentration of unoccupied paratopes will be the same in both reactions. For the reaction with soluble antigen, we get:

$$K_d(s) = A_{\text{free}}\,(B - C)/C,$$

and for the solid-phase reaction

$$K_d(sp) = A_{\text{free}}\,(F - E)/E.$$

Rearrangement gives

$$A_{\text{free}} = CK_{d(s)}/(B - C), and$$

$$A_{\text{free}} = EK_{d(sp)}/(F - E).$$

If the relative occupancy of paratopes on the solid-phase antigen is 0.5, then $E/(F - E) = 1$. We can then eliminate the terms E and F from the last equation:

$$A_{\text{free}} = K_{d(sp)}.$$

From the titrations we know how much higher the current dilution of serum is than that which gives antibody concentrations close to K_d: $A/K_{d(sp)}$; we call this ratio D, which is a dimensionless factor. Thereby we have an independent, alternative expression for$K_{d(sp)}$: A/D. We choose conditions such that the antigen in solution at 50% of maximal inhibition is in excess of the solid-phase anchored antigen. Therefore we let $A_{\text{free}} = A - C$ as an approximation.

We can now express $A - C = A/D \leftrightarrow C = A - A/D$.

The solution reaction is described by the equation $(A - C)(B - C)/C = K_{d(s)}$. $K_{d(s)}$ may differ from $K_{d(sp)}$: the reaction in solution may be essentially a monovalent binding, while the binding to solid phase is expected to be predominantly bivalent for IgG. The monovalent dissociation constants can be determined independently, by titrating the soluble antigen against immobilized antibody, or by titrating Fab against solid-phase antigen. However, in the competition assay the situation is complicated by the polyclonality of the antibodies in the serum: antigen molecules that have been complexed by one antibody may potentially bind to others bivalently. Furthermore the number of epitopes on an antigen molecule that on average would be occupied in the competition assay is not predictable. These factors constitute sources of limited uncertainty in this approach: the uncertainty can be resolved by calibration experiments with cocktails of monoclonal antibodies.

We can assume $K_{d(s)} = 5K_{d(sp)}$, which is reasonable based on the above considerations and previous knowledge of bivalent and monovalent binding (see, *e.g.*, (Chamow et al. 1990; and Klasse and Sattentau 1996). Furthermore we use $K_d(sp) = A/D$ and substitute $C = A - A/D$ into $(A - C)(B - C)/C = K_{d(s)}$. We get $A = B/6\big(1 - (1/D)\big)$ after simplification. In summary, we have derived a formula for estimating the concentration of paratopes from the concentration of competing antigen that is needed to halve binding in a competition ELISA. We have made use of a predetermined ratio of the serum dilution to that which gives antibody concentrations close to K_d. Hence the two

factors, antibody concentration and affinity, which together determine the antibody titer of a serum or plasma, can both be estimated by the combination of two kinds of ELISA.

A precondition for these calculations is an excess of antibody over antigen in the plasma under analysis, so that the original antigen in the plasma can be neglected in the competition assay. This condition has been fulfilled in analyses of plasma from HIV-1-infected persons (Binley et al. 1996). But what degree of complexing between antigen and antibody would occur *in vivo*? The considerations of different K_ds for different valencies apply here as well. Thus the K_d may be lower for bivalent binding to core particles than to monomeric p24. The K_d for binding to virion-bound antigen may be subject to the opposing influences of oligomerization and polyvalency. Antibody-antigen complexes may present polyvalent epitopes, and therefore have reduced K_d compared with unbound, monomeric antigen. Thus the respective values of K_d will require individual determination, but the occupancy on the antibody binding sites will equal

$$([Ab]/K_d)/(1 + [Ab]/K_d).$$

It is the ratio of the concentration of the more abundant molecule to its K_d, not the ratio of antibody to antigen concentration in plasma, that determines the degree of complexing. When plasma is diluted the complex formation gradually decreases, although the ratio of antigen to antibody by necessity would remain constant.

We have seen some instances of how the law of mass action governs antibody-antigen binding. But why are these relationships law-like and what do they tell us about the molecular structures involved in binding?

Binding energy. The affinity of a paratope for its epitope has its biophysical basis in the non-covalent bonds that are formed between the two surfaces (Barlow et al. 1986; Colman et al. 1987; Colman 1988). The hydrophobic effect is important in this regard, but so are hydrogen bonds and charge complementarity, particularly when surrounded by well-fitting surfaces in van der Waals contact (Creighton 1993; Janeway 1996). Thus, although the area of the epitope is related to the affinity, this is an approximate relationship (Barlow et al. 1986; Novotny et al. 1989). Affinity is another way of expressing the binding energy of the paratope-epitope reaction; it is the net result of all the forces acting on solutes and solvent. Affinity is thus a thermodynamic concept, and, if the concentration of free antibody is known, the dissociation constant can be translated into the difference in Gibbs free energy between bound and unbound antigen by the formula (Creighton 1993)

$$\Delta G_b = -RT\, ln([A]/K_d),$$

where $R =$ the general gas constant, $T =$ the absolute temperature, $[A] =$ the concentration of free antibody, and $K_d =$ the dissociation constant for the binding reaction. The binding energy says nothing about the rate at which the reaction occurs, only in which direction and to what degree it will proceed. For example, $\Delta G_b = 0$ when $[A] = K_d$: at equilibrium no further binding energy is released.

An alternative way of expressing the binding energy is

$$\Delta G_b = \Delta H - T\Delta S,$$

where ΔH and ΔS are the changes in enthalpy and entropy of the whole reaction system as binding occurs. If the epitope and paratope association raises the entropy of the surrounding water, by burial of hydrophobic surfaces around which the water otherwise forms ordered shells, this will contribute to the affinity (Creighton 1993; Janeway 1996). Likewise, if a paratope induces a conformational change in the antigen – within or outside the epitope – and the induced state is more relaxed or disordered, this would give higher affinity than if a more ordered state were induced. A high propensity of an epitope to be in the conformation that best fits the paratope is entropically more favorable than if the paratope has to induce, or select, an ordered conformation. As discussed below, this is relevant to the reciprocal binding effects that were found in a large study of cross-competition of MAbs to HIV-1 SU (Moore and Sodroski 1996).

Kinetics. The thermodynamic description of binding does not entail kinetic information about the reaction. But if the kinetic constants are known, the association and dissociation constants can be derived. As equilibrium is approached the rates of the backward and forward reactions denoted by the arrows in

$$A + B \rightleftharpoons AB,$$

get closer to identity. The rates are the products of the concentrations of the reactants and the rate constants. Thus the forward rate, or on-rate, is

$$[A][B]k_{\text{on}}\,,$$

and the backward rate, or off-rate, is

$$[AB]k_{\text{off}}\,.$$

Equilibrium is defined as the dynamic situation when

$$[A][B]k_{\text{on}} = [AB]k_{\text{off}}\,.$$

We see from this that for a simple bimolecular association reaction, the rate constants must have different dimensions. The k_{on} is expressed in units of $M^{-1}s^{-1}$ and k_{off} in units of s^{-1}. But the on and off rates are both measured in units of Ms^{-1}.

Furthermore it is apparent that the equilibrium constants can be expressed as ratios of the rate constants:

$$[A][B]k_{\text{on}} = [AB]k_{\text{off}} \Rightarrow$$

$$[AB]/[A][B] = k_{\text{on}}/k_{\text{off}} \Rightarrow K_a = k_{\text{on}}/k_{\text{off}}$$

$$[A][B]/[AB] = k_{\text{off}}/k_{\text{on}} \Rightarrow K_d = k_{\text{off}}/k_{\text{on}}\,.$$

This kinetic-thermodynamic relationship means that identical affinities of two antibodies can be the net result of very different kinetic binding properties. Currently, rate constants can be measured by the plasmon-resonance technique (Borrebaeck et al. 1992; Malmborg et al. 1992; VanCott et al. 1992; VanCott et al. 1994), which has been applied to recombinant Env-reactive Fab fragments produced by phage display (Roben et al. 1994). Thus the K_d or K_a can be calculated and compared with the value obtained by measuring equilibrium binding by ELISA. Good agreement between the two methods has been obtained for anti-Env Fabs (Roben et al. 1994). Phage display has also been used to increase the affinity of a MAb to V3 by finding antibody mutants with reduced k_{off} as observed by means of plasmon-resonance (Thompson et al. 1996). By the CDR-walking technique, as a further development of the phage-display method, the affinity of an anti-SU Fab was increased 420-fold. The resulting K_d was in the picomolar range. Selection was for ever increasing affinity for monomeric, immobilized SU; retrospectively the affinity increase was shown to be mediated mainly through a decrease in k_{off} (Yang et al. 1995). On- and off-rates for antibody binding to native oligomeric Env have been studied by FACS (fluorescence-activated cell sorting) analyses by varying the time allowed for antibody binding. The oligomerization of Env was found to affect mainly the on-rate (Sattentau et al. 1995).

As mentioned, both the hydrophobic effect and polar interactions contribute to the binding energy that determines the affinity. However, from a kinetic point of view these influences can be distinct: attractions between groups of opposite charges work over greater distances than van der Waals forces. Thus complementarity of charged groups may increase the affinity mainly by raising the on-rate constant. Conversely, large, well-fitting hydrophobic surfaces exert their strong influence on the binding after

water has been excluded, when the molecules are in apposition. This would tend to reduce the off-rate constant and hence increase the affinity (Colman 1988; Creighton 1993). But this dichotomy is an over-simplification, because charge complementarity makes its maximal contribution to the binding energy when the polar water molecules have been extruded, as is the case in the middle of well-fitting hydrophobic surfaces (Creighton 1993).

Antigenicity as a tool for probing three-dimensional structure

Can immunochemical data tell us anything about protein structure? We can distinguish three levels of knowledge of protein structure with decreasing degrees of certainty and precision: first, those structures determined down to a few Å resolution by crystallography or nuclear magnetic resonance; second, modeling based on suggested homologies with proteins of known structure; and, third, a combination of secondary-structural prediction and functional comparisons to a protein with known structure, complemented by a disulfide-bridge map, and mutational and immunochemical data. Some HIV and SIV proteins now fall in the first category: some have moved from the second to the first, while the Env protein remains in the third (Gallaher et al. 1995).

Eight-stranded β-barrels are well represented among virus proteins, and it was suggested that the major Gag protein of HIV-1, p24, might have this fold (Rossmann 1988). By the use of a sequence similarity of borderline significance between the picornavirus VP2 coat protein, whose structure is known, and the SIV major Gag protein, a model for the primate lentiviral major Gag proteins was advanced. This model consists of eight anti-parallel β strands forming a barrel, with a proline-rich protrusion or "puff", and an α-helix towards one end of the barrel (Argos 1989).

Overlapping nona-peptides covering the entire sequence of p24 were used to map continuous epitopes of murine MAbs, and of polyclonal antibodies from immunized sheep and rabbits as well as from HIV-1-infected humans. Superimposition of the epitopes on the VP2-based model of p24 predicted that they would be located on loops, turns and coils on the surface of the protein. Peptides derived from the anti-parallel β-strands in the model were poorly antigenic, but so were most of those representing parts of the putative puff region. These data were thought to corroborate the β-barrel model of p24 (Langedijk et al. 1990). But crystallographic analysis of a complex of a Fab with dimeric p24 revealed that part of the protein consists of seven α helices, five of which form a coiled coil (Momany et al. 1996). Some of the antigenic nona-peptides correspond to parts of these helices. Some antigenic sequences correspond to the face of p24 that is turned towards the other molecule in the dimer. The previously hypothesized exteriorly projecting puff region is partly α-helical in the crystal structure and faces the dimer interphase. This may contribute to its poor antigenicity. The Fab present in the complexes was derived from a MAb raised against disrupted virions. It is therefore as expected that it binds to the outer face of the dimer, which would be exposed on intact core particles.

The double reactivity of each of two MAbs with two nona-peptides which are 80 residues apart in the sequence is interesting (Langedijk et al. 1990). First, it illustrates that discontinuous epitopes can consist of antigenic structures which are sufficient for paratope binding on their own. Second, the apposition of the two sequences is not obvious from the crystal structure: they flank the E helix, but may be flexible (Momany et al. 1996). In theory, the identification of two antigenic structures far apart in a protein sequence that both can bind to the same paratope is strong direct evidence for the juxtaposition of those segments in the complexed form of the protein, while antigenic effects of mutations can always be subject to indirect effects. Nevertheless, the presence of the two segments in the epitope says nothing of how their proximity is created by the protein fold. In conclusion, although some topologies may be incompatible with certain immunochemical data, it is precarious to try to discriminate between a vast number of possible structures on the basis of such evidence.

The primary structure of HIV-1 SU can be divided into conserved and variable regions (Modrow et al. 1987). From the amino to the carboxy terminus these are: C1-V1-V2-C2-V3-C3-V4-C4-V5-C5. We do not know the structure of SU, but extensive knowledge of various proteins tells us that the variable regions have to be on the outside of the molecule, in the form of loops and turns. According to secondary-structural predictions, these would be connected both by β-strands and α-helices in SU (Gallaher et al. 1995). Do these connections form one or more cores in the protein? Most proteins that

have more than 200 residues (mature SU has around 480) are divided up into separate domains in the sense of compact structures that keep their fold when separated by proteolysis (Creighton 1993). The disulfide-bridge map of HIV-1 SU (Leonard et al. 1990) does not give strong clues as to whether SU has more than one domain in this sense: C1 is bridged to C2, which at the base of V3 is connected to C3, which is linked to C4. (The other disulfide bridges are within individual regions).

What do the extensive studies of MAb binding to wild-type and mutant SU tell us about its structure? Recently the binding of 46 MAbs to monomeric, native SU with the HXBc2 sequence was compiled into a cross-competition matrix (Moore and Sodroski 1996). Several of these MAbs are directed to discontinuous epitopes that have been mapped in mutational studies. If a mutation disrupts protein folding, it allows no inference about the direct involvement of the mutated residue in a discontinuous epitope. Thus mutational studies may only identify a subset of residues with direct antigenic relevance. In HIV-1 SU there are epitopes which are sensitive to mutations in, and thus may include residues of, the following pairs of regions: C1 and C4, C1 and C5, C2 and C3, C4 and V3, and C3 and V4. The TM-interactive region includes parts of C1 and C5. The CD4-binding site may involve residues in C2, C3 and C4 (refs in (Moore and Sodroski 1996)). Epitopes on SU that are induced by the binding of CD4 are sensitive to changes in most regions (Thali et al. 1993), which increases the probability that some mutational effects are indirect. Nevertheless, the pattern of extensive overlap from epitope to epitope fails to indicate any division of SU into distinct domains. Likewise, the competition of the MAbs to continuous and discontinuous epitopes shows an intricate web of inhibition and enhancement. Some effects are reciprocal, others not (Moore and Sodroski 1996). The cross-competition patterns can roughly be categorized into clusters of epitopes, but even within these there is sometimes enhancement of the binding of one antibody by another. There are effects across clusters, which suggest an intimate conformational interdependence of different parts of the monomer rather than division into separate domains.

The frequent finding of non-reciprocality in effects on binding is challenging. It is worth noting that although the structural inferences from such data can only be crude, their relevance, at least for passive immunization, may go far beyond that of structure determinations at atomic resolution: it is not yet possible to predict how the binding of a MAb to one epitope on a three-dimensional model of a protein will affect the antigenicity other parts. The answer to such questions requires the cross-competition approach, whether the structure of the protein is known or not. Further, cross-competition data may remain the only basis for discussing the structure of the Env protein for some time to come. Hence it is pertinent to ask what reciprocal and non-reciprocal inhibition of MAb binding could mean in physico-chemical terms.

The competition matrix was generated by measuring the effects of the pre-binding of an unlabeled MAb on the subsequent binding of a labeled MAb. The MAbs included in the study have affinities for native SU that fall in a narrow range (Moore and Sodroski 1996). Thus, when a 50% decrease in binding of a labeled MAb at subsaturating concentrations results from the prebinding of another, unlabeled MAb, and the two MAbs can bind simultaneously to the same SU monomer, this means a few-fold reduction in the affinity of the labeled MAb. If the reduction in binding of the labeled MAb is from half-maximal to 25% of maximal binding this means a tripling of K_d (Figure 1). The most straightforward logical relationship is between reciprocal inhibition of two MAbs and the overlap of their epitopes: if essential components in the two epitopes overlap, that implies incompatibility of binding, which should manifest itself as reciprocal inhibition. Unfortunately, the implication in the opposite direction would be more useful. But if the unlabeled antibody is used at concentrations many-fold above its K_d and the labeled one is not, enhancement implies non-overlap.

The increase in antigenicity of the epitope of the labeled MAb could be mediated by an allosteric conformational change induced by the unlabeled MAb. A continuum of four kinds of mechanisms of increased antigenicity may be hypothesized. We call the epitope of the labeled MAb L, and of the unlabeled MAb U.

1) The exposure of L could be augmented, *e.g.*, by the drawing in of an obtruding flexible loop into U.

2) When a MAb binds to U, L may switch from one inflexible conformation to another which better fits the paratope.
3) If L is normally flexible, the binding of a MAb to U may lock it in a conformation that favors binding to the paratope.
4) If L is normally locked in a conformation that disfavors binding, the binding of a MAb to U may increase flexibility of L and thereby its antigenicity.

It is apparent from the previous section that, all other things being equal, the first mechanism could decrease the K_d by increasing the apparent k_{on} . The last two would reduce K_d by distinct enthalpic and entropic contributions to the free energy of the binding to L. The most plausible explanation for the increase in the antigenicity of one epitope by the binding of a MAb to another may vary from case to case. For example, the V2 region is unlikely either to be buried or to have a rigid, regular secondary structure because it is known to accommodate extreme variability. Furthermore, mutual enhancement of the binding of some V2-directed MAbs was seen (Moore and Sodroski 1996). These factors taken together would fit well with stabilization as the mechanism of enhancement of antigenicity, possibly for mutual enhancement of binding to V2 and V3 as well. C1- and C5-directed MAbs also showed more than average mutual enhancement of binding. The amino- and carboxy-terminal regions are often flexible in proteins (Creighton 1993). If that is so in SU, these regions may also have potential for stabilization, although by virtue of their high conservation they may form regular secondary structures.

The greatest explanatory challenge is posed by the finding that some MAbs enhanced the binding of those they were themselves blocked by. For example, a MAb to a discontinuous epitope in C1-C4 enhances the binding to CD4-induced epitopes, while a MAb to this latter kind of epitope inhibits the binding of the MAb to the C1-C4 epitope. Bivalent soluble CD4 (CD4-IgG) enhances the binding of the same MAb to the C1-C4 epitope, while this MAb strongly inhibits the binding of CD4-IgG. If the induction of greater flexibility or rigidity by the binding to two different sites is reciprocal, but the one epitope is favored by flexibility, the other by rigidity, as in the third and fourth mechanism above, then reciprocal conformational effects would give non-reciprocality in antigenic terms.

The immunochemical studies on SU structure can take advantage of a criterion that is unavailable when the Gag structure is probed: whether an antibody neutralizes viral infectivity or not. SU is a major target for such antibodies, and the reasonable assumption is that an epitope capable of binding such antibodies must be presented on SU in its TM-anchored, oligomeric form on infectious virions. The salient feature of the model resulting from the competition matrix is the distinction of two faces of one compact structure: a smaller face that contains residues implicated in hetero-oligomerization with TM and a larger one that presents neutralization epitopes.

Neutralization of HIV-1

Virus neutralization is the reduction in infectivity that results from the binding to the virus particles of antibodies or soluble forms of receptors for the virus. This definition thus includes any abrogation of infectivity by the binding of antibodies to cellular antigens present on the surface of the virions, as has been described for primate-lentiviruses (Arthur et al. 1992). The definition does not include the action of antibodies that reduce HIV-1 infectivity by binding to the cellular receptors for the virus, such as certain antibodies to domains 1, 2, and 3 of CD4 (reviewed in (Klasse et al. 1993; Weiss 1993), or as future research may show, antibodies to the chemokine receptors that serve as co-factors for HIV infection (Alkhatib et al. 1996; Alkhatib et al. 1996; Deng et al. 1996; Doranz et al. 1996; Dragic et al. 1996; Feng et al. 1996). The definition leaves open whether neutralization is reversible (Layne et al. 1991; Merges et al. 1994) or irreversible (Layne et al. 1991; McDougal et al. 1996), and whether it is restricted to viral infectivity of certain cell types or blocks the entire cellular tropism of the virus (McEntee et al. 1992; McKnight and Clapham 1995; McKnight et al. 1995), and by what mechanism it works. The measurement of neutralization requires the application of an infectivity assay. We can distinguish three processes, not necessarily separate in time, in such an experimental system:

1) The binding of the antibody to the virus particles. If whole serum or plasma is used, the concentration of the antibodies can potentially be estimated as described above. If only the plasma

dilution is known, it simplifies if this is expressed as parts per volume, as this is proportional to the concentration, while the dilution factor, *e.g.* one-hundred-fold, bears a hyperbolic relationship to the concentration.

Only a small fraction of the virus particles in an HIV-1 inoculum will infect the target cells (Layne et al. 1992; Dimitrov et al. 1993; Klasse and McKeating 1993). The defects of the non-infectious particles are multifarious, *e.g.* too few SU per virion, inactive reverse transcriptase, and defective genomes (Layne et al. 1992). The infectivity of a viral stock varies with temperature and duration of inoculation, as well as with type and density of target cells (Layne et al. 1991). Furthermore, most of the infectious virions in an HIV-1 inoculum do not adsorb to highly susceptible cells (Kabat et al. 1994). Thus, many potentially infectious virions do not infect. The terminology in this area can be confusing. For example, it has been stated that V3-specific antibodies in the sera of symptomatic HIV-1-infected persons are directed to non-infectious and not to infectious virions (Schreiber et al. 1994). "Non-infectious" in this case would include virus that has been neutralized. In order to avoid misunderstanding, we can make the distinction between non-infectious (inert), non-infecting (but potentially infectious) and infecting virus. Neutralizing antibodies may bind to virus in all three categories but can neutralize only when they bind to virus in the latter two, which is detected as an effect on virus in the third category.

The binding of neutralizing antibody to virus occurs in accordance with the law of mass action, and classically, this reaction has been allowed to approach equilibrium before the target cells are exposed to virus. The affinity and concentration of the neutralizing agent will then determine the relative occupancy on the neutralization epitopes according to the formula $([A]/K_d)/(1 + [A]/K_d)$ (Klasse and Moore 1996).

However, in studies of HIV-1 neutralization by soluble CD4, it was argued that in order to improve the neutralization assay as a model of processes that may occur *in vivo*, cells should be present from the start (Layne et al. 1990; Layne et al. 1991). This makes the binding of the antibodies overlap in time with process number 2, below.

2) The adsorption of virus to susceptible cells. This process is potentially also amenable to modeling in accordance with the law of mass action (Klasse and Moore 1996): a virus particle, albeit with different diffusion characteristics from those of a globular protein, will have a certain affinity for its cellular receptors. However, the HIV-1 particles would have heterogeneous affinities for cells because of variable numbers of SU per virion. We do not know the affinity of whole HIV-1 virions for susceptible cells yet, although such measurements of the binding of MuLV virions have been made (Yu et al. 1995).

We can let virus that has been pre-incubated with antibody adsorb to cells; or we may reverse the chronology of process 1 and 2 by finding a temperature that is permissive for virus adsorption but not for fusion or entry. HIV-1 adsorbs but does not fuse at temperatures below 25° C (Fu et al. 1993; Frey et al. 1995). Thus preincubating the target cells with virus at, for example, 4° C, washing off unbound virus and then adding antibody, will allow neutralization of virus that was adsorbed to cells. Furthermore, the target cells can be warmed up for variable periods before the antibody is added; thereby the relative kinetics of the binding of the neutralizing antibody and the process it interferes with can be assessed (Lu et al. 1992; Pelchen-Matthews et al. 1995).

3) Replication of virus that has not been neutralized. Some effect of the replication, such as production of viral protein, proviral DNA or syncytium formation, has to be quantitated. One approach that allows the detection of a single cycle of viral replication is the measurement of the activation by the viral Tat protein of the bacterial *LacZ* gene, which has been placed under the control of the viral LTR in the target cells (Charneau et al. 1992). In another approach, defective recombinant virus, which contains the gene for an enzyme, is used (Thali et al. 1994; Sullivan et al. 1995; Karlsson et al. 1996). Some assays involve the counting of infectious units, some use end-point dilutions of virus infectivity, still others rely on measurements of continuous biochemical quantities. Neutralization is often expressed as the percent reduction from the signal obtained without antibody. Such a percentage can mean different things. It is clearest what it means if the infectious dose, *i.e.*, the amount of the suspension of viral particles, is proportional to the infectivity signal after subtraction of background (Figure 2).

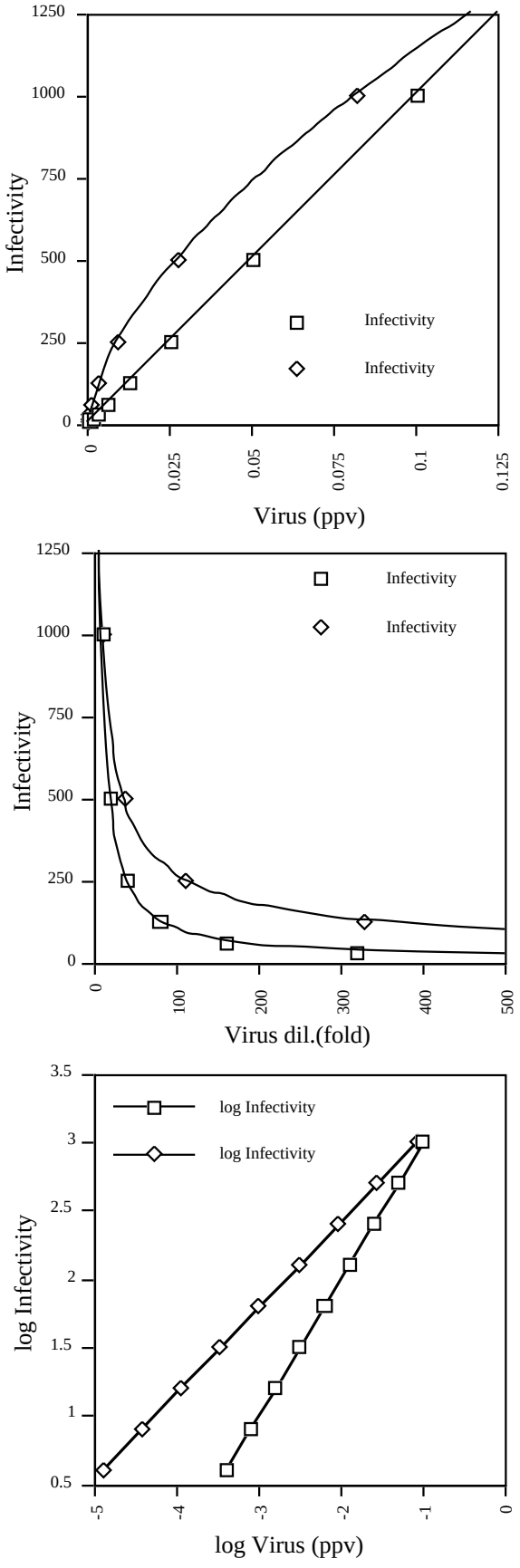

Figure 2. In a neutralization assay that demonstrates what proportion of the infectious virions is neutralized, the infectivity as a function of input viral dose is a proportionality, *i.e.*, a linearity that goes through the origin. (a) If the volume of the inoculum is kept constant, the viral dose would correspond to the concentration of the virus in the inoculum expressed as parts per volume (ppv). A linear relationship is shown for one virus (open squares), and a non-linear one for another virus or the same virus in another assay (open diamonds). (b) If infectivity is plotted as a function of dilution of the viral stock, a hyperbolic and not a linear relationship is expected. Both the linear and non-linear curves in (a) are converted to hyperbolas. This plot does not distinguish between linear and non-linear dose-infectivity relationships. (c) A linear relationship, which does not demonstrate proportionality, is obtained by a plot of the logarithm of infectivity as a function of logarithm of the viral dose. Both data sets from (a) give linearity in this analysis.

Focus- or plaque-counting assays may seem to come closest to measuring infectious units directly. But these assays may require safeguards to secure proportionality: agarose overlays or inhibitors of reverse transcription to prevent progeny virus from yielding secondary foci. The need for such precautions is obviated by the use of a defective recombinant virus.

Other assays make use of the counting of syncytia, or better the nuclei in syncytia, that arise from infection with a certain dose of virus. It is apparent that the fusion between infected and uninfected cells bears no simple relationship to the number of preceding infectious events. Likewise the commonly measured production of viral p24 can be the complex result of many generations of propagating progeny virus.

A simple experiment can verify that the infectivity as a function of the amount of virus added is a proportionality (Figure 2). A hyperbolic relationship of the infectivity to the dilution factor of the inoculum is expected (Figure 2), although linearity in a zone of such a plot has been observed for HIV-1 infectivity measured as syncytium formation (McLain and Dimmock 1994). Figure 2 also illustrates that proportionality is not demonstrated by representing the logarithm of infectivity as a function of the logarithm of the viral dose.

Post-neutralization infectivities can be plotted as I/I_o, *i.e.*, the infectivity resulting from incubation of the virus with a certain neutralizing antibody divided by the control infectivity obtained by incubation with an inactive antibody. Only when there is proportionality between amount of input virus and infectivity readout, or signal, will 50% reduction in signal mean that 50% of the infectious virions have been neutralized; only then will 75% reduction in signal mean that three times more virions have been inactivated than after 25% reduction. For many assays both of these statements are false. A sinister possibility is that two viral strains deviate in different ways from proportionality in an assay (cf. Fig.2), for example if the infectivity signal is affected by cytotoxicity. 50% reduction in signal for both will then correspond to different degrees of neutralization. The proportionality is also important for differentiating between synergistic and additive neutralizing effects of antibodies and soluble receptors (see *e.g.* Allaway et al. 1993)).

The kinetics and molecularity of neutralization. If process 1, antibody binding to virus, can be quenched before the start of process 2, i.e. virus adsorption to the target cells, then the kinetics of the neutralization reaction itself can be studied. We can answer such questions as: How much more neutralization has occurred after one hour than after 20 minutes? Dulbecco et al. applied this kind of analysis to the neutralization of poliovirus and Western Equine Encephalitis virus (Dulbecco et al 1956). They plotted the rate of neutralization as a function of the concentration of the neutralizing antibody. They found that the neutralization reaction was of the first order *in antibody concentration*, $[A]^1 k$.

However, they interpreted this as meaning that the neutralization was a single-hit phenomenon, in other words that only one antibody molecule was required to neutralize one infectious virion. The number of molecules involved in a chemical reaction is its *molecularity*. The kinetics and molecularity of a reaction must be kept apart, for although single-hit molecularity implies first-order kinetics in the concentration of each reactant, the reverse is not true (Atkins 1986). Thus, first-order kinetics in antibody concentration does not imply single-hit molecularity of neutralization. But the number of hits required for neutralization can be translated into a relative occupancy of antibodies on the virion-associated Env protein for a stipulated number of SU molecules per virion (Klasse and Moore 1996). As shown above, the occupancy can be estimated when there is antibody excess and the K_d for the binding to the relevant oligomeric form of Env is known. However, some antibodies to SU, to the V2 and V3 regions, but not to the CD4-binding site, have been shown to induce the dissociation of SU from TM (Poignard et al. 1996), similarly to the effect of soluble CD4 (Moore et al. 1990). This complicates the determination of the number of hits required for neutralization (Klasse and Moore 1996): the number of SU per virion remaining after the induced shedding would need to be known in order to determine the molecularity. More problematic is the heterogeneity of virion populations and the difficulty of demonstrating that measurements of SU number per virion are representative of the fraction of virions that are potentially infectious. This heterogeneity would also impede the determination of the stoichiometry, or molecularity, by the use of radioactively labeled antibody as applied to the neutralization of polio and influenza virus (Icenogle 1983; Taylor 1987).

In stoichiometric analyses, the plot of the logarithm of relative infectivity is more informative than for investigating the proportionality of viral dose dependence, as shown in Figure 2: if the binding of antibodies is Poisson-distributed, and the neutralization is a single-hit reaction, then the negative natural logarithm of the relative infectivity will equal the average number of antibodies per virion. Poisson analysis of HIV-1 neutralization is discussed elsewhere (Klasse and Moore 1996). The major point is that few-hit neutralization would correspond to very low relative occupancies of antibodies on virions, and even lower for greater numbers of intact Env oligomers per virion. It is plausible that virions that have shed all but a minimal number of SU required for infection will be neutralized by the binding of a few IgG molecules. But for a virion with, for example, 100 SU monomers the binding of one IgG molecule would constitute a relative occupancy of 2%. This occupancy would be obtained by a concentration of antibody 49-fold below its K_d. Higher antibody concentrations are usually required for HIV-1 neutralization, yet single- and few-hit neutralization by MAbs to HIV-1 SU has been inferred from kinetic data (McLain and Dimmock 1994).

The kinetics of the neutralization reaction must not be confused with the kinetics of antibody binding, which can theoretically be of another order. Nevertheless, the kinetics of antibody binding is highly pertinent to neutralization. The lower degree of binding of some MAbs to oligomeric than to monomeric Env may be the result of a lower on-rate constant (Sattentau and Moore 1995). A MAb with lower on-rate constant than another gives a lower pre-equilibrium occupancy, even if the two MAbs have the same affinity for an epitope. As outlined above, the duration of the pre-incubation of virus with antibodies may determine the influence of such kinetic factors on the outcome of a neutralization experiment. When virus, neutralizing agent and cells are mixed simultaneously (*e.g.* Layne et al. 1991), this kinetic influence is maximized.

Neutralization escape and resistance. When HIV-1 is propagated in the presence of neutralizing antibody, resistant viral variants arise. This is neutralization escape. Many cases of viral escape from neutralizing antibodies highlight the need for regarding epitopes as structures in three-dimensional space. The first neutralization-escape mutant of HIV-1 was obtained by propagating the molecular clone HxB2D of the T-cell line-adapted isolate LAI-IIIB in the presence of a serum from an HIV-1-positive person (Robert-Guroff et al. 1986). The resistance to neutralization was due to a mutation in the extravirional part of gp41, the phylogenetically most conserved region of the whole of Env: Ala 582 → Thr (Reitz et al. 1988). The mutant is neutralized less efficiently than wild-type by approximately one third of HIV-1-positive human sera (Wilson et al. 1990). The antigenicity, but not the propensity to helicize, of TM-derived peptides that encompass Ala 582 is greatly reduced by the Ala → Thr substitution (Klasse et al. 1993). However, the antibodies reactive with such peptides do not neutralize the virus (Wilson et al. 1990). Rather, MAbs to SU epitopes that overlap with the CD4-binding site neutralize the wild-type more efficiently than the mutant (Klasse et al. 1993; Thali et al. 1994). Such a difference was observed with MAbs obtained by immunization with recombinant SU, but to a greater extent with human MAbs (Klasse et al. 1993). It is conceivable that the surfaces of the affected epitopes are close in space to residue 582. But a substitution that confers neutralization resistance to foot-and-mouth-disease virus shows that the residue responsible for the escape phenotype need not be close to the epitope even in three-dimensional space. In that case the change of a Thr to an Ala reduces the affinity of the neutralizing MAb, although the mutated residue is not in spacial proximity to the epitope (Parry et al. 1990).

The HxB2-Env:Ala 582 → Thr mutant has a greater capacity to induce syncytia than wild-type virus or a spontaneous revertant, which has the additional mutation Ser673 → Phe (Stern et al. 1995). This could mean that the process that neutralization blocks is mediated more swiftly by mutant than revertant or wild-type Env. Nevertheless, the escape phenotype is very specific and does not affect neutralization mediated by antibodies to V3 (Reitz et al. 1988; Klasse et al. 1993), soluble CD4 or the neutralization epitope that includes residues 667–672 in TM (Klasse et al. 1993).

Another experiment in which serum from an HIV-1-infected person was used to select variants of HxB2 also resulted in a neutralization-escape mutation in a conserved region: an Ala → Val substitution at position 282, which is in the C2 region. The change conferred strong resistance to the selecting serum, but only marginal relative resistance to other sera from infected individuals; the mutant was as sensitive

as wild-type to MAbs directed to the CD4-binding site, but somewhat more resistant to a MAb directed to V3, which is close in sequence to the mutation (Watkins et al. 1993).

Immune-selection with a MAb to the V3 region gave rise to a mutant with a V3 amino-acid sequence that decreased the binding of the MAb to the mutant peptide compared with the wild-type peptide (McKeating et al. 1989). A V3 mutant of HIV-1IIIB arose *in vivo* in the laboratory worker who was accidentally infected with this isolate. This mutation specifically conferred resistance to a V3-directed MAb, and decreased the binding of the MAb to native SU, while the corresponding substitution in synthetic peptides had no effect on antigenicity (di Marzo Veronese et al. 1993). Immune-escape from V3-specific neutralizing antibodies in experimentally infected chimpanzees has occurred by amino-acid changes outside V3 (Nara et al. 1990). Resistance to V3 MAbs, and to some extent to MAbs to the CD4-binding site, of HIV-1IIIB variants that arose in chimpanzee infection has been mapped to TM (Back et al. 1993).

Mutants of the HIV-1 isolate RF, which escaped neutralization by a MAb to a conformationally sensitive epitope in V2, showed a reduced affinity of the MAb for native SU. The resistant phenotype was demonstrated to be caused by the substitution Tyr 177 → His (Yoshiyama et al. 1994). In summary, many different kinds of structural and functional effects of neutralization-escape mutations have been observed; the examples given, as well as other possibilities, are categorized in Figure 3.

Primary isolates (PI) are generally less sensitive to neutralization than strains which are adapted to growth in T-cell lines (TCLA) (Moore and Ho 1995; Poignard et al. 1996; Sattentau 1996). It is noteworthy that in this case the genetic relationship is the converse of that of the escape variants and their parental strains: the more neutralization-sensitive TCLA were ultimately derived from more resistant PI. The PI-TCLA difference is an instance of relative neutralization resistance, of which neutralization escape is special case. In order to explain such resistance, we must express it quantitatively. If we let $[A]_1$ be the minimal concentration required to neutralize a defined fraction of a certain dose of the more sensitive virus, and $[A]_2$ the corresponding one for the same dose of the more resistant virus, we can express the resistance as the ratio $[A]_2/[A]_1$, which is > 1 (Figure 3). For PI versus TCLA neutralization by soluble CD4 and MAbs $[A]_2/[A]_1$ is often around 1000. Since resistance may be multi-factorial, it is imperative to demonstrate how much of the ratio a certain mutation or property is responsible for.

A mathematical model of HIV-1 neutralization resistance has been presented elsewhere (Klasse and Moore 1996). This model describes a relationship between neutralization resistance and the affinity of the neutralizing antibody or soluble receptor for the oligomeric form of the Env protein on the virion. Further, it incorporates the total number of SU per virion and the minimal number of SU-TM molecules needed for the attachment to and fusion with susceptible cells. The model predicts that a greater difference between total and minimal SU numbers for one virus than for another, all other things being equal, will result in relative neutralization resistance. PI have been found to have a several-fold greater ratio of virion-bound SU over Gag than TCLA (references in Moore and Ho 1995, and Klasse and Moore 1996), but at present it cannot be demonstrated that such differences exist for the infectious fractions of the virus, nor that the excess of total SU over the number minimally required is greater for PI than TCLA. It was recently shown by the use of recombinant PI virions with low average SU content per virion that a greater SU number per virion is not a necessary factor in making PI more neutralization-resistant than TCLA (Karlsson et al. 1996). It has been possible to vary the SU number of TCLA while keeping most other factors constant by allowing spontaneous shedding of SU from virions for different periods. Sensitivity to neutralization by soluble CD4 was found to correlate with increased losses of SU (Layne et al. 1991; Layne et al. 1992). Because of the lower degree of spontaneous shedding from PI virions (Moore et al. 1992; Moore et al. 1993; Moore and Ho 1995), other experiments will have to be designed to investigate what effect variation in SU number has on their neutralization sensitivity.

It is hypothesized that the binding of CD4 to the Env oligomers can lead either to the activation of fusion or to an abortive dissociation of SU from TM (Moore et al. 1990; Sattentau and Moore 1991; Klasse and Sattentau 1996; Poignard et al. 1996; Poignard et al. 1996). The temperature and pH dependences of productive and abortive CD4 interactions are distinct (Fu et al. 1993). Some MAbs, to the V2 and V3 regions of SU can also induce SU dissociation from TM on TCLA virus (Poignard et al. 1996).

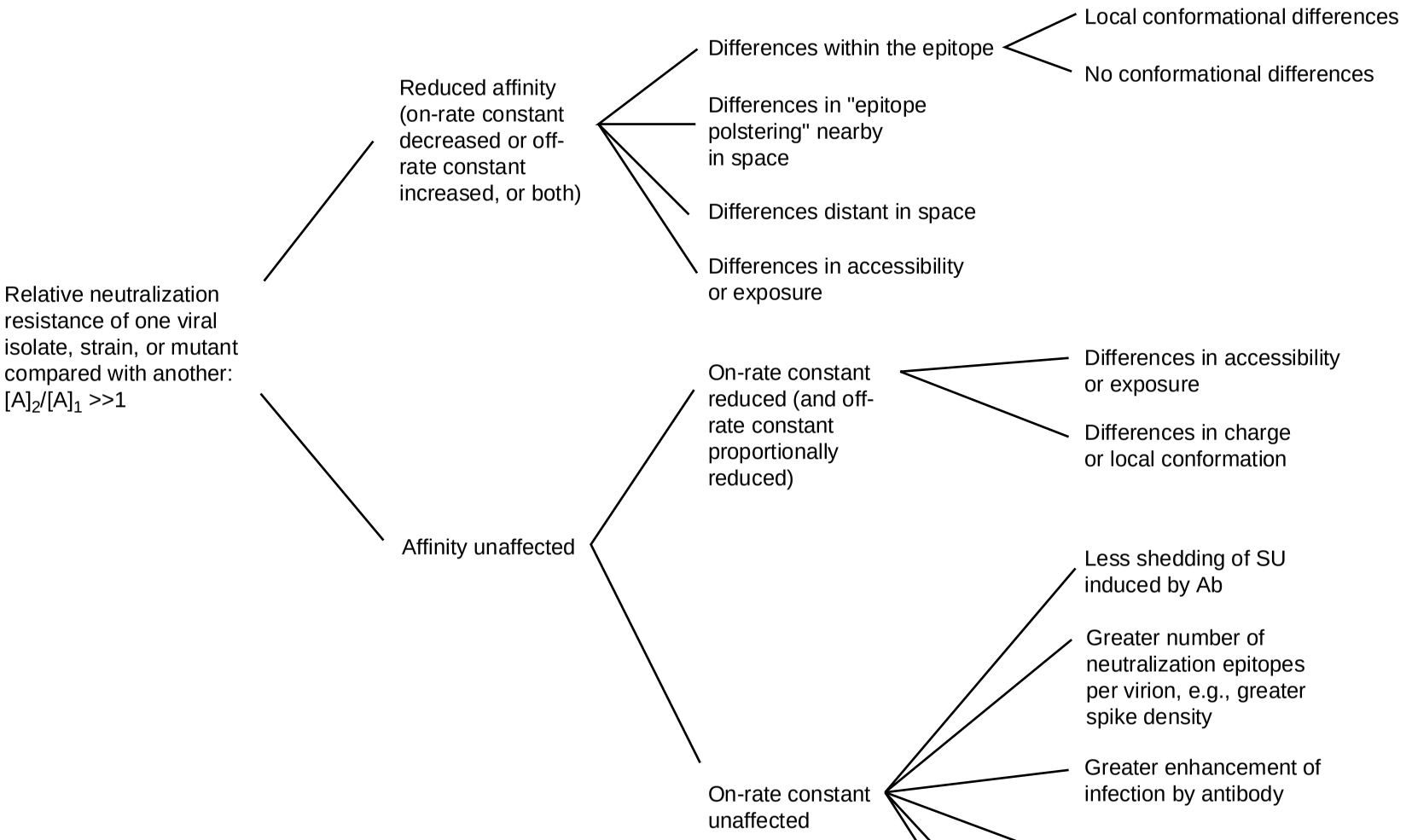

Figure 3. Definition and categorization of relative neutralization resistance and its causes. Each branch represents a pure mechanism as a causative factor in the resistance. Some categories are mutually exclusive, others can plausibly be combined in creating resistance. Examples of most categories can be found in references in the text, whereas some mechanisms lack experimental support at present.

However, both soluble CD4 and some anti-SU MAbs can enhance PI infectivity (Schutten 1995; Sullivan et al. 1995; reviewed in Poignard et al. 1996). These phenomena may be explained by a lowering of the activation energy of fusion and SU shedding by the binding of the MAb or CD4 (Klasse and Moore 1996). The lowering of the threshold for fusion may be part of the escape mechanism of the HxB2-Env:Ala 582 $\rightarrow$ Thr mutant (Stern et al. 1995), which is resistant to antibodies of CD4-binding site-specificity (Klasse et al. 1993; Thali et al. 1994). Such antibodies were found not to induce SU dissociation from a related TCLA clone. This is in contrast to V3-specific MAbs (Poignard et al. 1996) and soluble CD4, to which the mutant remains sensitive. In general, there may be a subtle balance between a lowering of the threshold for fusion and increased susceptibility to agents that induce SU shedding (Klasse and Moore 1996). An analysis of antibody occupancy on virions is central to the understanding of neutralization resistance. But variations in the processes that neutralization interferes with have to be considered as well (Figure 3).

Conclusions

In the course of HIV-1 infection in humans, the antibodies produced change in isotype composition, concentration, affinity and neutralizing capacity. The antigenic properties of HIV-1-derived peptides differ from the corresponding epitopes on proteins. Furthermore, monomeric and oligomeric forms of the native proteins differ in antigenicity, as has been shown in greatest detail for the Env proteins. Antigenicity, in particular the mapping of discontinuous epitopes, can provide clues to protein topology. MAb cross-competition can yield information about conformational interdependence of epitopes that goes beyond what could be inferred from high-resolution structures.

Affinities can be quantitated by applications of the law of mass action. On- and off-rate constants have been measured for the binding of antibodies to HIV-1 antigens. From these measurements the equilibrium association and dissociation constants can be derived, while the converse is impossible. Serum or plasma titers are determined by the concentrations of antibodies and their dissociation or association constants in accordance with the law of mass action, which can be applied to measurements of antibody concentrations and average affinities in serum or plasma samples. The design of a virus-neutralization assay can maximize the influence of either the affinity or the on-rate constant of the neutralizing antibody. The degree of occupancy of antibodies on the virions can be calculated from the ratio of antibody concentration over the dissociation constant for antibody binding to virions.

Neutralization resistance can theoretically be determined by antibody affinity for oligomeric Env, the affinity of the virion for the cell, the number of epitopes per virion that minimally need to be blocked, and the energy thresholds of activation of fusion and of abortive conformational changes in Env.

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